EDGEWOOD CHEMICAL BIOLOGICAL CENTER
U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1395

DARPA ANTIBODY TECHNOLOGY PROGRAM

STANDARDIZED TEST BED FOR ANTIBODY CHARACTERIZATION: CHARACTERIZATION OF AN MS2 SCFV ANTIBODY PRODUCED BY ILLUMINA

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August 2016

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-08-2016	Final	Oct 2010 – Sep 2012

4. TITLE AND SUBTITLE
DARPA Antibody Technology Program
Standardized Test Bed for Antibody Characterization: Characterization of an MS2 ScFv Antibody Produced by Illumina

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Director, ECBC, ATTN: RDCB-DRB-M, APG, MD 21010-5424
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8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-1395

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Advanced Research Project Agency, 675 North Randolph Street, Arlington, VA 22203-2114

10. SPONSOR/MONITOR'S ACRONYM(S)
DARPA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
The selection of antibodies for inclusion in a final assay format has primarily relied on an antibody's performance in an enzyme-linked immunosorbent assay, with little regard for quantification of the full spectrum of variables affecting antibody–antigen interactions. The Joint Product Management Office for Biosurveillance (JPMO BSV) Critical Reagents Program has recently instituted a quality program for standardization of test methods to support more comprehensive characterization and comparison of the physical and functional properties of antibody reagents within its repository. The development and standardization of antibody testing provides JPMO BSV with an invaluable platform for the provision of consistent, high-quality assays and reagents for current biodetection platforms as well as the development and validation of future systems. This platform will be used to characterize and evaluate the MS2 recombinant antibody produced and supplied by the Defense Advanced Research Project Agency-funded investigator Illumina (San Diego, CA) for both affinity and stability enhancements. The results of this study not only provide standardized parametric data on antibody properties and performance, but also contribute to the development of a decisional analysis tool for expanding the confidence level of antibody-based reagent selection to optimize field operational and performance metrics of future detection and diagnostic platforms.

15. SUBJECT TERMS

Antibody	Antibody Technology Program (ATP)
Quality	Enzyme-linked immunosorbent assay (ELISA)
Biosurveillance	Single-chain fragment variable (scFv)
MS2 coat protein (MS2CP)	

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Renu B. Rastogi
U	U	U	UU	32	19b. TELEPHONE NUMBER *(include area code)* (410) 436-7545

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PREFACE

The work described in this report was supported by Defense Advanced Research Projects Agency funding. The work was started in October 2010 and completed in September 2012.

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STANDARDIZED TEST BED FOR ANTIBODY CHARACTERIZATION: CHARACTERIZATION OF AN MS2 SCFV ANTIBODY PRODUCED BY ILLUMINA

1. INTRODUCTION

Current platforms for detection and diagnosis of biothreat agent exposure depend on the use of antibodies to recognize and bind to specific antigens. To date, the selection of antibodies for inclusion in a final assay format has primarily relied on an antibody's performance in an enzyme-linked immunosorbent assay (ELISA), with little regard for quantification of the full spectrum of variables affecting antibody–antigen interactions. The Joint Product Management Office for Biosurveillance (JPMO BSV) Critical Reagents Program (CRP) instituted a quality program for the standardization of test methods to more fully characterize and compare the physical and functional properties of antibody reagents in its repository. The development and standardization of antibody testing provides the JPMO BSV with an invaluable platform for the provision of consistent, high-quality assays and reagents for existing biodetection platforms and also for the development and validation of future systems. This platform will be used to characterize the MS2 single-chain variable fragment (scFv) antibody produced at Illumina (San Diego, CA) for the Defense Advanced Research Projects Agency (DARPA) Antibody Technology Program (ATP).

The DARPA ATP focuses on developing technologies for enhancing the thermal stability and binding affinity of a given antibody. Functioning as an independent testing laboratory for this program, the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) has provided specific technical support on immune reagents and defined the government-supplied antibody–antigen pairs. The goal of this project was twofold: (a) select, develop, and standardize the methods for characterizing the de novo thermal and binding properties of select reagents to be used by DARPA-funded investigators, and (b) use those methods to validate the changes in antibody thermal stability and binding affinity that were achieved by the DARPA investigators. The antibody chosen for this project was the MS2 recombinant scFv produced at ECBC (*1*), which detects an MS2 coat protein (MS2CP) that forms the capsid for the MS2 bacteriophage. The focus of the work highlighted in this report is the evaluation of the MS2 antibodies supplied by the DARPA-funded investigator Illumina for both affinity and stability enhancements. The results of this study not only provide standardized parametric data on antibody properties and performance, but also contribute to the development of a decisional analysis tool for expanding the confidence level during selection of antibody-based reagents that will optimize the field operational and performance metrics of future detection and diagnostic platforms.

2. MATERIALS AND METHODS

2.1 MS2 ScFv and MS2CP

MS2 scFv antibody was produced from a plasmid supplied by Ellen Goldman (U.S. Naval Research Laboratory; Bethesda MD). The plasmid was designated Gv1, and the sequence was cloned into a pET-22b(+) plasmid (EMD Millipore; Billerica, MA). The protein was produced and eluted in 20 mM sodium phosphate (pH 8.0), 0.5 M sodium chloride, and 0.5 M imidazole. Peak fractions were then collected and separated on a 16/60 Superdex 200 gel filtration column (GE Healthcare Life Sciences; Pittsburgh, PA), and fractions that corresponded with a monomeric protein were collected and flash-frozen in liquid nitrogen. These fractions were then provided (along with sequence data) to Illumina as baseline material.

The MS2CP was produced from a pET-28a(+) plasmid (Novagen; Madison, WI): the MS2CP sequence was inserted with an amino acid substitution of an arginine at position 83 in a construct engineered by DNA2.0 (Menlo Park, CA). MS2CP was produced and eluted with 300 mM imidazole in pH 7.4 phosphate-buffered saline (PBS; Sigma-Aldrich Company; St. Louis, MO). Peak fractions were collected, and buffer was exchanged into PBS (pH 7.4) using a 470 mL packed volume of Sephadex G-25 fine gel chromatography media (Amersham Biosciences Corporation; Piscataway, NJ). The fractions were provided to Illumina as antigen for the MS2 antibody.

2.2 UV-Visible Spectrophotometry

A NanoDrop ND-1000 spectrophotometer (Thermo Scientific; Waltham, MA) was used to determine the MS2 scFv concentrations and the absorbance of light at 280 nm (A_{280}) for the samples supplied by Illumina. The A_{280} value is influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient is used in conjunction with the A_{280} value to determine an accurate concentration. The MS2 scFv concentrations were determined by dividing the average A_{280} value by 1.77, which is the extinction coefficient for a scFv. Each reading required a 2 µL sample, which was placed on the sample pedestal. The arm of the instrument was lowered, creating a liquid column between the top of the arm and the surface of the pedestal; this was the path length through which the laser passed. The instrument was blanked using PBS, and readings were taken in triplicate. A positive control, bovine γ-globulin (BGG; Bio-Rad; Hercules, CA), was also tested to validate the instrument operation.

2.3 Electrophoresis

Molecular weight and purity data were collected with an Experion automated electrophoresis system (Bio-Rad). The system employs microfluidic technology to automate electrophoresis for protein analysis. The microfluidic chip, in conjunction with the Experion reagents, electrophoresis station, and software, are designed to accomplish separation, staining, destaining, detection, and basic data analysis. The Experion Pro260 analysis kit uses engineered lower and upper internal alignment markers to provide clean baselines, accurate molecular weight sizing, and quantitative protein analysis (*2*). The Pro260 analytical software also

determines sample purity by calculating the percent mass of the separated proteins in a sample. For Experion analysis, each of the Illumina MS2 scFvs was standardized to a final concentration of 1 mg/mL by diluting it in PBS. The BGG control and the Illumina samples were then processed using a validated procedure specified in the Bio-Rad Experion Pro260 analysis kit, rev. C (*3*). Briefly, a Pro260 microfluidic chip was prepared by adding 12 µL of Pro260 gel and gel stain to the designated wells. The chip was then placed on the priming station and primed for 1 min at the medium (B) pressure setting. The priming filled the fluidic channels with gel, which was used by the instrument to form a barrier between samples during the run. The sample was reduced with dithiothrietol (Sigma-Aldrich) and denatured in the kit-provided sample buffer at 95 °C before it was applied to the primed chip. The chip was then placed in the instrument, and the lid was closed, lowering the sample needles into the wells. The instrument was operated via the Experion software; each chip took 30 min to complete. All samples were run in triplicate alongside one sample of the BGG control and the Pro260 ladder. All analysis was performed using the Experion software.

2.4 Dynamic Light Scattering (DLS)

DLS was used to paint a picture of how the proteins behaved in solution. DLS data indicates whether a protein is in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. Prediction algorithms within the software produce a range of values for the protein under evaluation. For DLS analysis, five 20 µL aliquots of the Illumina MS2 scFvs, along with the control bovine serum albumin (Sigma-Aldrich), were placed into a quartz 384-well plate (Wyatt Technology Corporation; Santa Barbara, CA) and centrifuged for 2 min at 239 ×*g* to remove trapped air bubbles from the samples. Mineral oil (Sigma-Aldrich) was applied to the top of each sample to prevent sample evaporation. The plate was placed in a DynaPro temperature-controlled plate reader (Wyatt Technology). Each well was scanned 10 times for 5 s each at 25 °C. Values were averaged to provide measurements of polydispersity, hydrodynamic radius, percent mass, and molecular weight for each sample using Dynamics software (Wyatt Technology). The results of three wells were averaged and reported.

2.5 Differential Scanning Calorimetry (DSC)

DSC was used to obtain a quantitative melting temperature (T_m) for each of the Illumina MS2 scFv proteins. The T_m should predict the results of subsequent ELISA and surface plasmon resonance (SPR) thermostability testing. A T_m above 70 °C predicts that the percent of antibody activity after the thermal stress test will remain above 50%. A T_m below 70 °C predicts at least a 50% decrease in antibody activity after the thermal stress test. For DSC experiments, samples were diluted to 0.5 mg/mL and dialyzed overnight in PBS (pH 7.4). Samples were degassed for 5 min before analysis and injected into the sample cell of a VP-DSC microcalorimeter (MicroCal; Northampton, MA). Dialysis buffer was added to the reference cell of the calorimeter, and a buffer scan was used as the baseline for all experiments. The samples (in duplicate) were scanned from 15 to 100 °C at a rate of 60 °C/h. The transition midpoint of the protein was determined by data analysis using Origin 7.0 software (MicroCal).

2.6 Thermal Stress Test

All samples were diluted to 1 mg/mL before heat was applied to negate protective effects due to concentration (*2*). The Illumina thermally stabilized antibody (AFX-733) was diluted to 1 mg/mL in 1× PBS and divided into five tubes. One aliquot was kept on ice for the duration of the experiment and was marked time 0. The remaining four aliquots were heated to 70 °C on a calibrated heat block for 15, 30, 45, and 60 min each. After each time point, the corresponding aliquot was removed and placed in an ice bath. These samples were then tested for activity.

2.7 ELISA

ELISAs were performed in triplicate using standard techniques. After the thermal stress test, each sample was diluted to 1 µg/mL in PBS and used to coat one row each of three Nunc MaxiSorp 96-well plates (Thermo Scientific), and incubated at 4 °C overnight. In the morning, each plate was washed in 1× wash buffer (KPL; Gaithersburg, MD) using a standard wash protocol on an AquaMax 200 plate washer (Molecular Devices; Sunnyvale, CA). The plate was blocked with 1× milk diluent block (MDB) (KPL) for 30 min at 37 °C. The plate was washed, and PBS with 0.05% Tween 20 (PBS-T; Sigma-Aldrich) was applied to the plate such that each well received 100 µL. MS2CP was diluted in PBS-T to 2 µg/mL, and 100 µL was applied to the first well of each row. A twofold serial dilution was performed across the plate, and it was incubated for 1 h at 37 °C. After the plate was washed, mouse anti-MS2 (kindly supplied by the JPMO BSV CRP) was diluted to 5 µg/mL in 1× MDB, and 100 µL was added to each well. The plate was incubated at 37 °C for 1 h. The plate was washed, goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP) (KPL) was diluted to 0.2 µg/mL in 1× MDB, and 100 µL was added to each well. The plate was incubated at 37 °C for 30 min. After the plate was washed, 100 µL of room-temperature 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) 1-component HRP substrate (KPL) was added to each well. After 20 min at 37 °C, the optical density at the 405 nm light wavelength was determined using a Synergy H4 hybrid multi-mode microplate reader (BioTek; Winooski, VT). Data analysis was performed using Prism software (GraphPad Software; La Jolla, CA).

2.8 SPR Methodology

One method for determining the kinetic parameters of antibody–antigen interactions is SPR. This is a rapid methodology for monitoring biomolecular interactions through excitation of surface plasmons. Polarized light is shone through a prism on a sensor chip with a thin metal film coating, which reflects the light by acting as a mirror. If the angle of light shone through the prism is changed, and the intensity of the reflected light is monitored, differences in intensity can be recorded. While the refractive index at the prism side of the chip is not changing, the refractive index in the immediate vicinity of the metal surface will change when accumulated mass (bound proteins) adsorbs on the surface. Therefore, if binding occurs, the resonance angle (SPR angle) changes, and this SPR angle shift provides information on the protein adsorption kinetics on the surface. The software can then provide an accurate analysis of the association (k_a) and dissociation (k_d) rate constants for the antibody interactions, as well as calculate the overall affinity constant (K_D) between antibody and antigen.

2.8.1 Thermostability Testing Using SPR

A Biacore T200 system (GE Healthcare) and standard amine coupling chemistry were used to tether 6500 response units (RUs) of MS2CP to one flow cell of a Biacore CM5 sensor chip. After a thermal stress test was performed, samples were centrifuged at 2000 ×*g* and 5 °C for 5 min. The analyte was run at 10 µL/min for 120 s. A calibration curve was created by injecting eight concentrations of the time 0 unheated Illumina MS2 scFv samples (AFX-733 or AFX-719) at 400, 350, 300, 250, 200, 150, 100, and 50 nM and plotting the respective analyte-binding capacity of the surface (R_{Max}) in response units. Unheated and heated samples were then diluted 1:90 and 1:180 in order for the time 0 control points to fall on the linear calibration curve. All samples were run in triplicate. The chip's surface was regenerated with an 18 s injection of 0.85% phosphoric acid at a flow rate of 30 µL/min. Data was collected using the Biacore concentration analysis software, and the active concentration of heated sample was recorded. The running buffer used for this experiment was Biacore HBS-EP 1× buffer (GE Healthcare Life Sciences).

2.8.2 Kinetic Analysis Using SPR

A ProteOn XPR36 SPR system (Bio-Rad), PBS-T running buffer, and standard amine coupling chemistry were used to tether 200 RU of MS2CP to a GLC sensor chip (Bio-Rad). Illumina MS2 scFv samples (AFX-733 or AFX 719) were injected across the chip's surface for 120 s at a flow rate of 100 µL/min with a 600 s dissociation at 5 nM, 1.67 nM, 560 pM, 190 pM, and 60 pM. The chip's surface was regenerated using an 18 s injection of 0.85% phosphoric acid at 100 µL/min. Data was analyzed using a Langmuir 1:1 fit.

3. RESULTS

3.1 Spectrophotometry Results

Both of the Illumina MS2 scFvs were read in triplicate on the NanoDrop ND-1000 spectrophotometer. The A_{280} readings are listed in Table 1.

Table 1. NanoDrop A_{280} Readings

Replicate No.	A_{280} Reading (mg/mL)
AFX-733	
1	4.135
2	4.138
3	4.056
AFX-719	
1	4.423
2	4.961
3	4.497

For each antibody, these three numbers were averaged and divided by the extinction coefficient of 1.77. The final concentrations were determined to be 2.32 mg/mL for the thermally stabilized AFX-733 and 2.62 mg/mL for affinity matured AFX-719.

3.2 Electrophoresis Results

The molecular weights of the Illumina MS2 scFvs were determined using the Experion Pro260 analysis kit, as shown in Figure 1. The thick bands in the second and fourth lanes correspond to the MS2 scFv antibodies AFX733 and AFX719, respectively. According to the Experion software, AFX-733 was 88.4% pure and weighed 35.8 kDa, and AFX-719.14 was 82.6% pure and weighed 37.1 kDa.

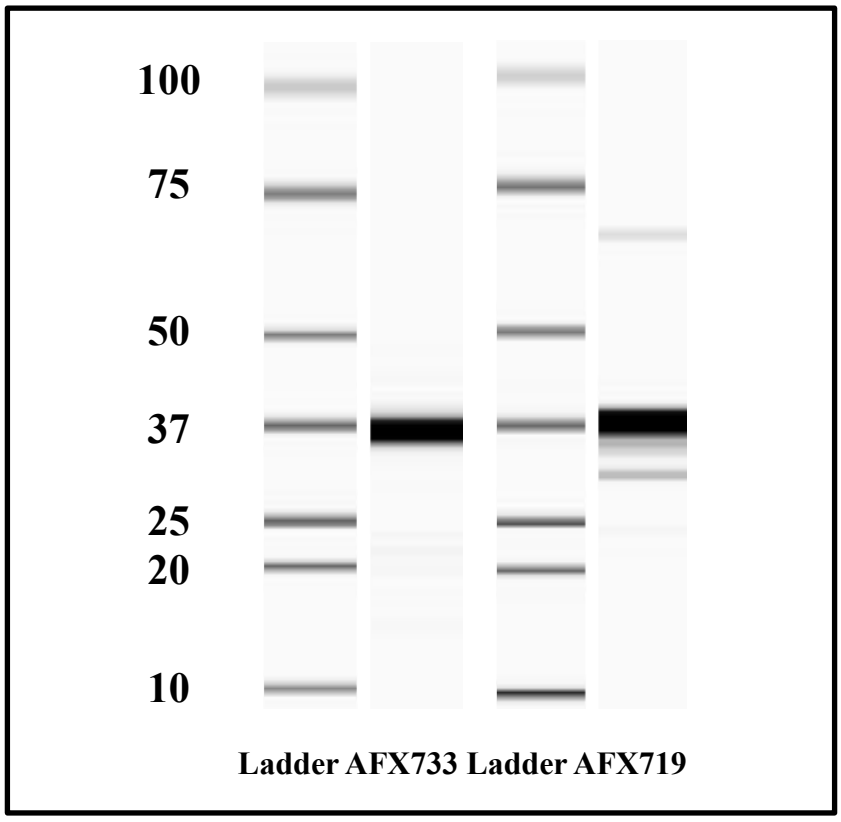


Figure 1. Molecular weight and purity. Digital gel of Illumina MS2 scFvs produced using the Experion Pro260 analysis kit. The thick bands in the second and fourth lanes correspond to the MS2 scFv antibodies AFX733 and AFX719, respectively.

3.3 DLS Results

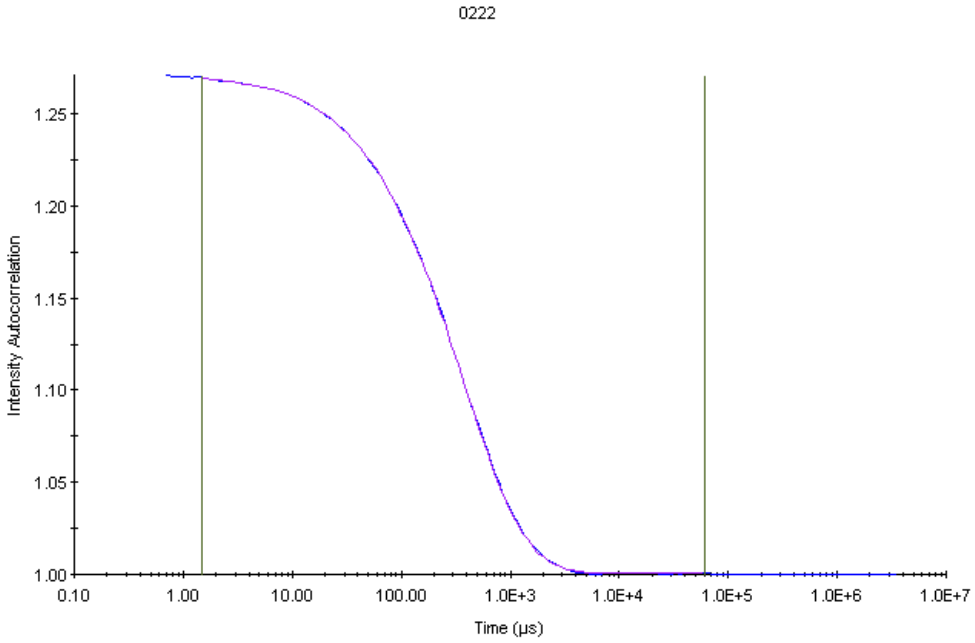
Both of the Illumina MS2 scFvs were analyzed in triplicate using the DynaPro plate reader. The radius of AFX-733 was determined to be 3.0 nm with a polydispersity of 14.7% (Table 2), whereas the radius of AFX-719 was determined to be 5.84 nm with a polydispersity of 54.8%. Figure 2 contains representative correlation and regularization graphs for each of the

MS2 scFvs. The correlation graphs (Figures 2A and 3A) depict a sigmoidal curve indicative of a valid size distribution. The regularization graphs (Figures 2B and 3B) illustrate the monodispersity found in both samples. Table 2 shows the raw data produced for each replicate. Because 89.9 or 99.9% of the mass displayed favorable polydispersity and hydrodynamic radius, both of these sample preparations were considered to be monodisperse.

Table 2. Features of MS2 ScFv in Solution

Sample	Replicate	Radius (nm)	Polydispersity (%)
AFX-733	1	2.7	24.0
	2	3.4	11.8
	3	2.8	16.3
	4	3.4	11.6
	5	2.8	9.9
	Average	3.0	14.7
AFX-719	1	6.0	55.7
	2	6.4	64.5
	3	5.9	54.7
	4	5.1	44.2
	Average	5.8	54.8

A. Correlation graph of AFX-733



B. Regularization graph of AFX-733

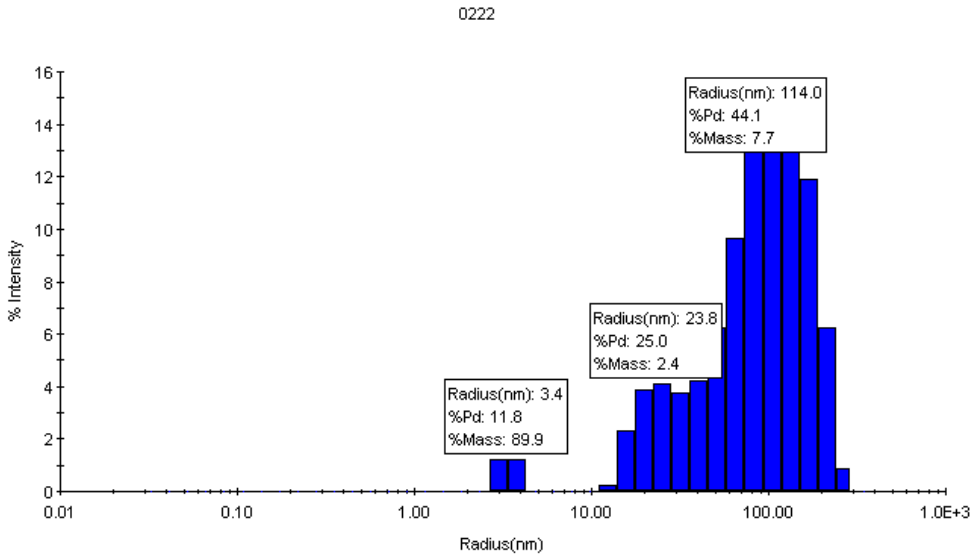
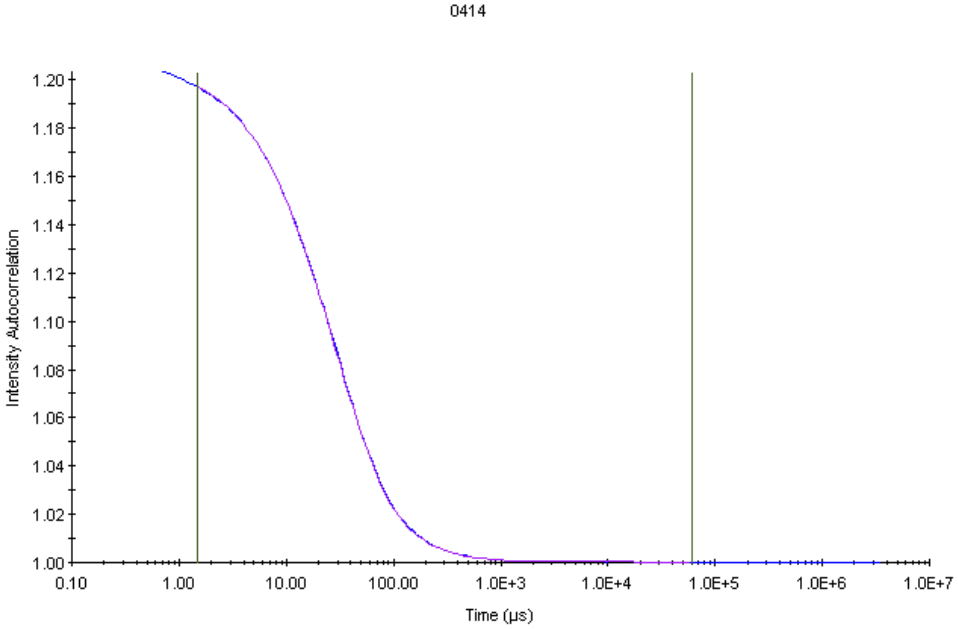


Figure 2. Radius and polydispersity representation of thermostable scFv AFX-733.
A: Correlation graph; and **B:** regularization graph of the Illumina thermostable scFv AFX-733.

A. Correlation graph of AFX-719



B. Regularization graph of AFX-719

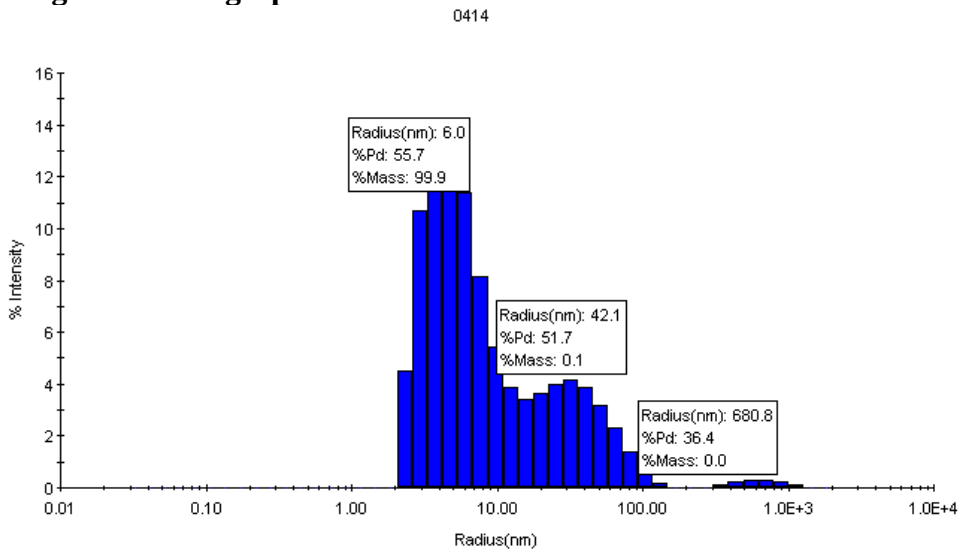


Figure 3. Radius and polydispersity representation of affinity matured scFv AFX-719. **A:** Correlation graph; and **B:** regularization graph of the Illumina affinity matured scFv AFX-719 used for determining the radius and polydispersity of the samples.

3.4 DSC Results

The Illumina MS2 scFvs were read in duplicate on the MicroCal VP-DSC. The peak T_m values were as follows:

- AFX-733 Replicate 1: 73.6 °C;
- AFX-733 Replicate 2: 73.6 °C;
- AFX-719 Replicate 1: 66.3 °C; and
- AFX-719 Replicate 2: 66.7 °C.

The final T_m for AFX-733 was determined to be 73.6 °C (Figure 4), and that for AFX-719 was determined to be 66.5 °C (Figure 5).

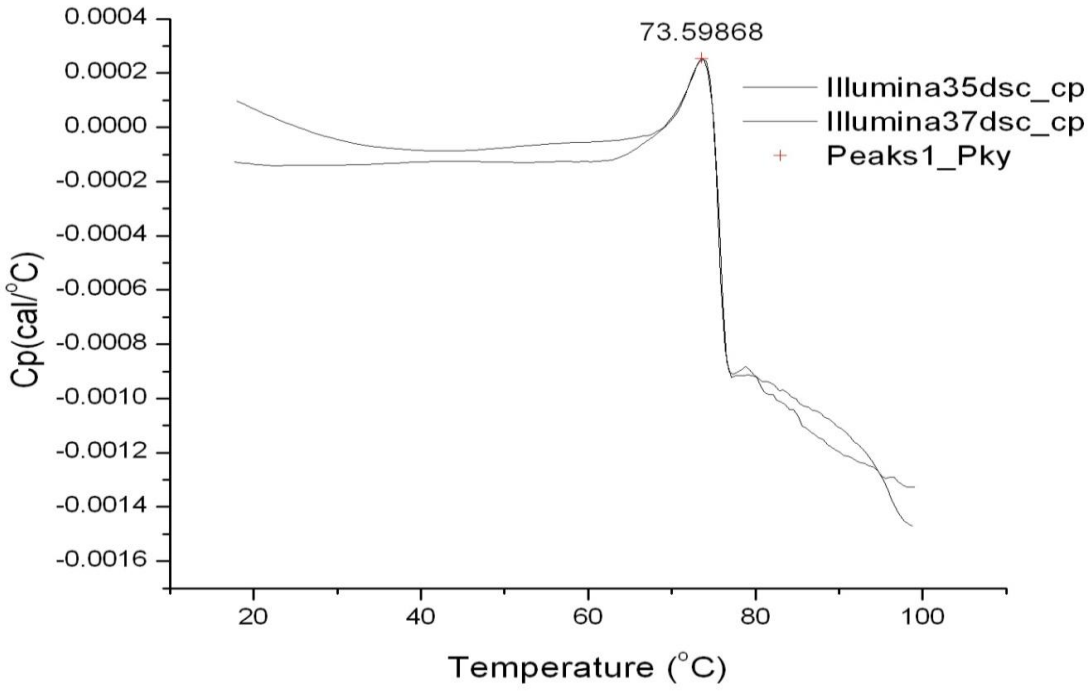


Figure 4. Transition midpoint curve for AFX-733. Curves were generated on the MicroCal VP-DSC microcalorimeter and analyzed using the Peak Find function in the Origin 7.0 software. The transition midpoint was calculated to be 73.6 °C for AFX-733.

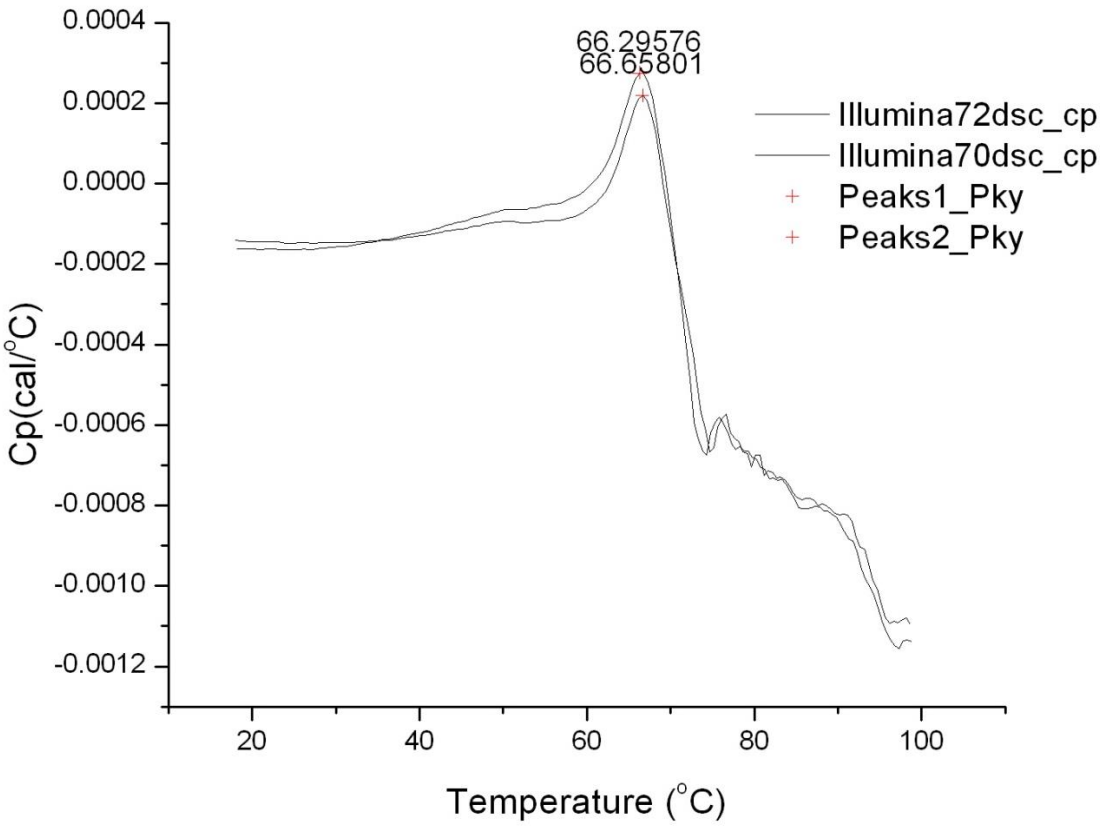


Figure 5. Transition midpoint curve for AFX-719. Curves were generated on the MicroCal VP-DSC microcalorimeter and analyzed using the Peak Find function in the Origin 7.0 software. The transition midpoint was calculated to be 66.5 °C for AFX-719.

3.5 ELISA Results

ELISA assays were used to test the functional interaction of antibody and antigen after thermal stress at 70 °C. The ELISA data (Figure 6) show that when the Illumina scFv AFX-733 was heated to 70 °C, it maintained all activity across all time periods of thermal stress, unlike the government-supplied MS2 scFv. The curves in Figure 6A show antibody activity for different time points at 70 °C as a function of the concentration of antigen supplied. The area under the curve for each of the different time points at 70 °C was calculated, averaged, and graphed to depict how the MS2 scFv reacted over time to thermal stress. The graph in Figure 6B illustrates that all of the MS2 scFv remained functionally capable of binding to antigen after a 60 min exposure to 70 °C, unlike the original scFv, which lost activity within 15 min of heating.

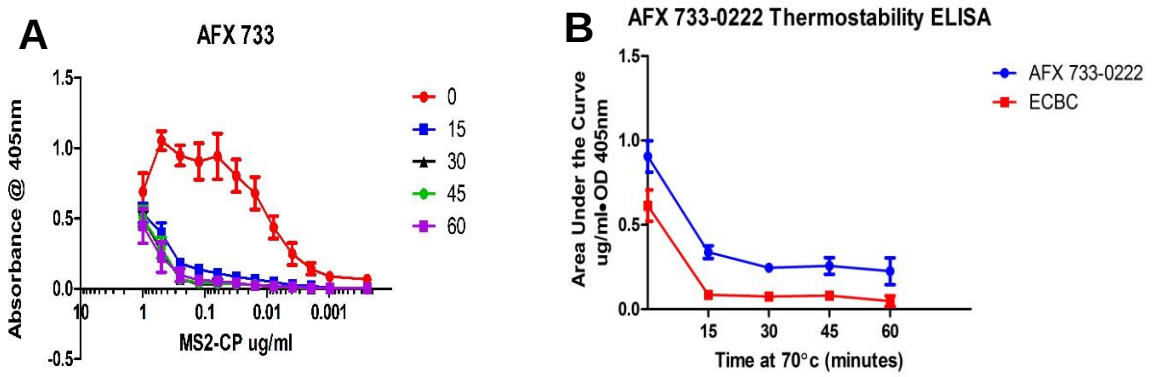


Figure 6. Thermostability of Illumina AFX-733 using ELISA.
A: Activity of AFX-733 as a function of antigen concentration after thermal stress. The different curves are represented on the legend by the number of minutes the sample was held at 70 °C.
B: Area under the curve analysis depicting the effect of thermal stress.

3.6 SPR Results

3.6.1 Thermostability Testing Results Using SPR

SPR was used to assess the functional binding between the Illumina MS2 scFv (AFX-733) and the antigen after the antibody–antigen complex was heated to 70 °C for variable time periods. Five tubes of 1 mg/mL MS2 scFv were prepared and heated to 70 °C for the following time periods: 15, 30, 45, and 60 min, followed by quenching on ice. A Biacore T200 system was used to compare the activity of each sample with a calibration curve for unheated sample. The percent activity of the heated samples was plotted over time (Figure 7). The results indicate that the scFv remained active over the entire 60 min; whereas, the activity of the scFv dropped off completely within the first 15 min at 70 °C.

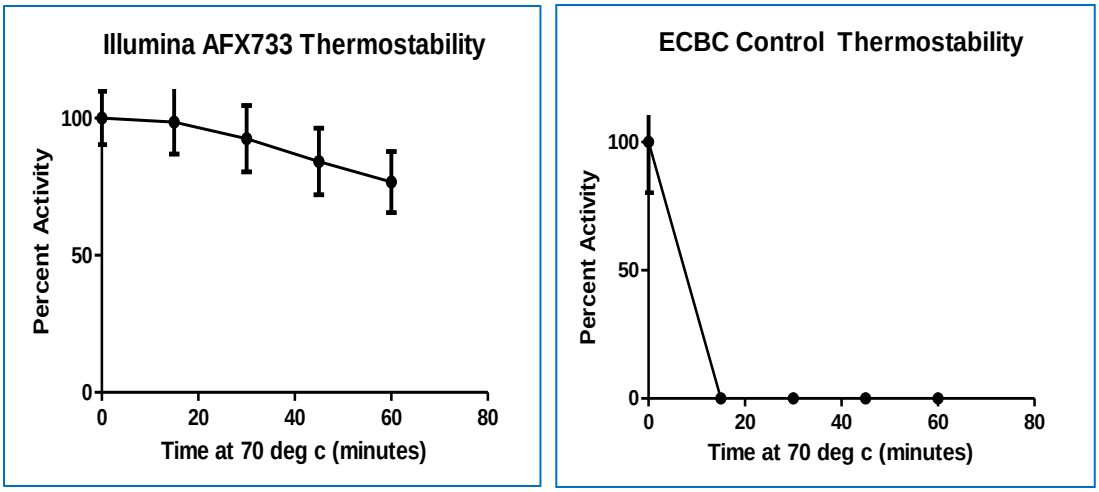


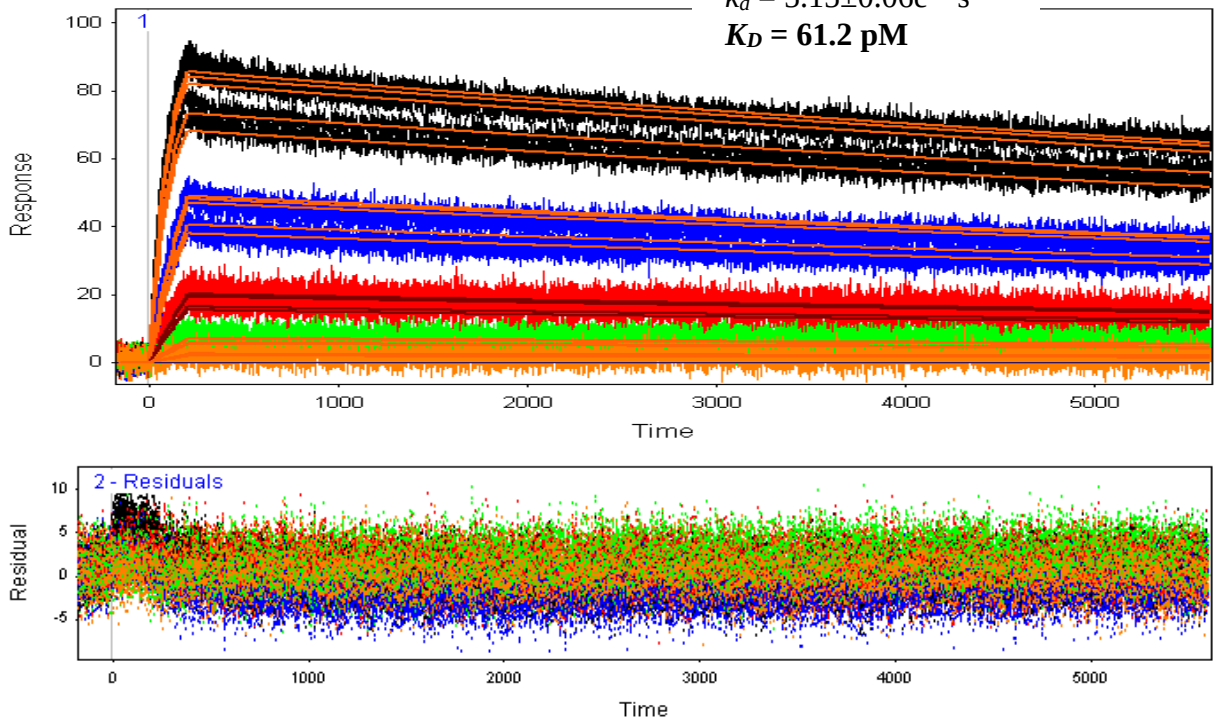
Figure 7. Thermostability of the Illumina MS2 scFv (AFX-733; left) compared with the ECBC MS2 scFv by SPR (right). The Illumina antibody maintained over 85% of its activity after heating to 70 °C for 60 min, compared with the original ECBC MS2 scFv antibody, which lost all ability to recognize the MS2CP target within 15 min of heating.

3.6.2 Kinetic Analysis Results Using SPR

Kinetic analysis of the affinity-enhanced Illumina MS2 scFv (AFX-719) binding to the MS2CP antigen was performed as a direct-binding SPR experiment on the Proteon XPR36, and the results are presented in Figure 8A. Data were normalized to a blank-immobilized reference flow cell and fit to a Langmuir 1:1 model using the Bio-Rad Proteon XPR36 software. The K_D was determined to be 61.2 pM. Similar experiments run using the original ECBC MS2 scFv are presented in Figure 8B. The K_D of the original scFv was determined to be 8.66 nM; thus, Illumina provided an antibody that was well above the 100-fold improvement threshold. Finally, kinetics analysis was also performed on the thermostable-enhanced Illumina MS2 scFv (AFX-733), and the results are presented in Figure 8C. The K_D was determined to be 8.5 nM.

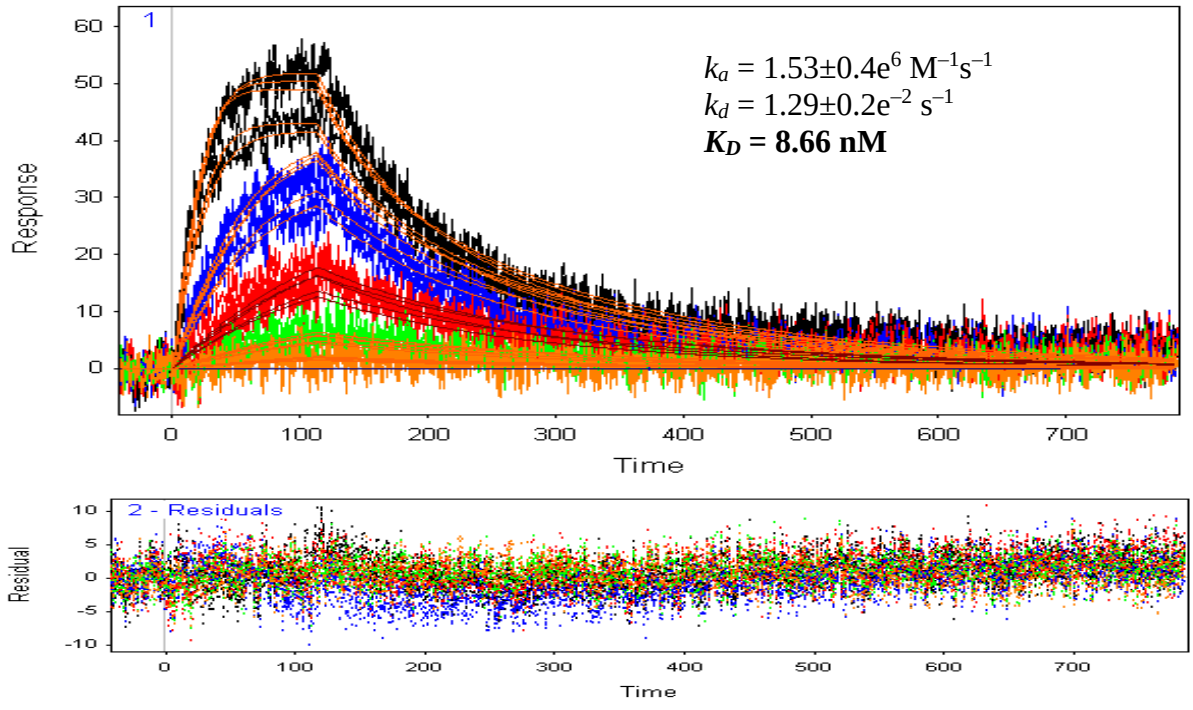
8-A. Illumina AFX-719

k_a = 8.43±0.4e^5 M^{-1}s^{-1}
k_d = 5.15±0.06e^{-5} s^{-1}
K_D = 61.2 pM



8-B. ECBC MS2 scFv

k_a = 1.53±0.4e^6 M^{-1}s^{-1}
k_d = 1.29±0.2e^{-2} s^{-1}
K_D = 8.66 nM



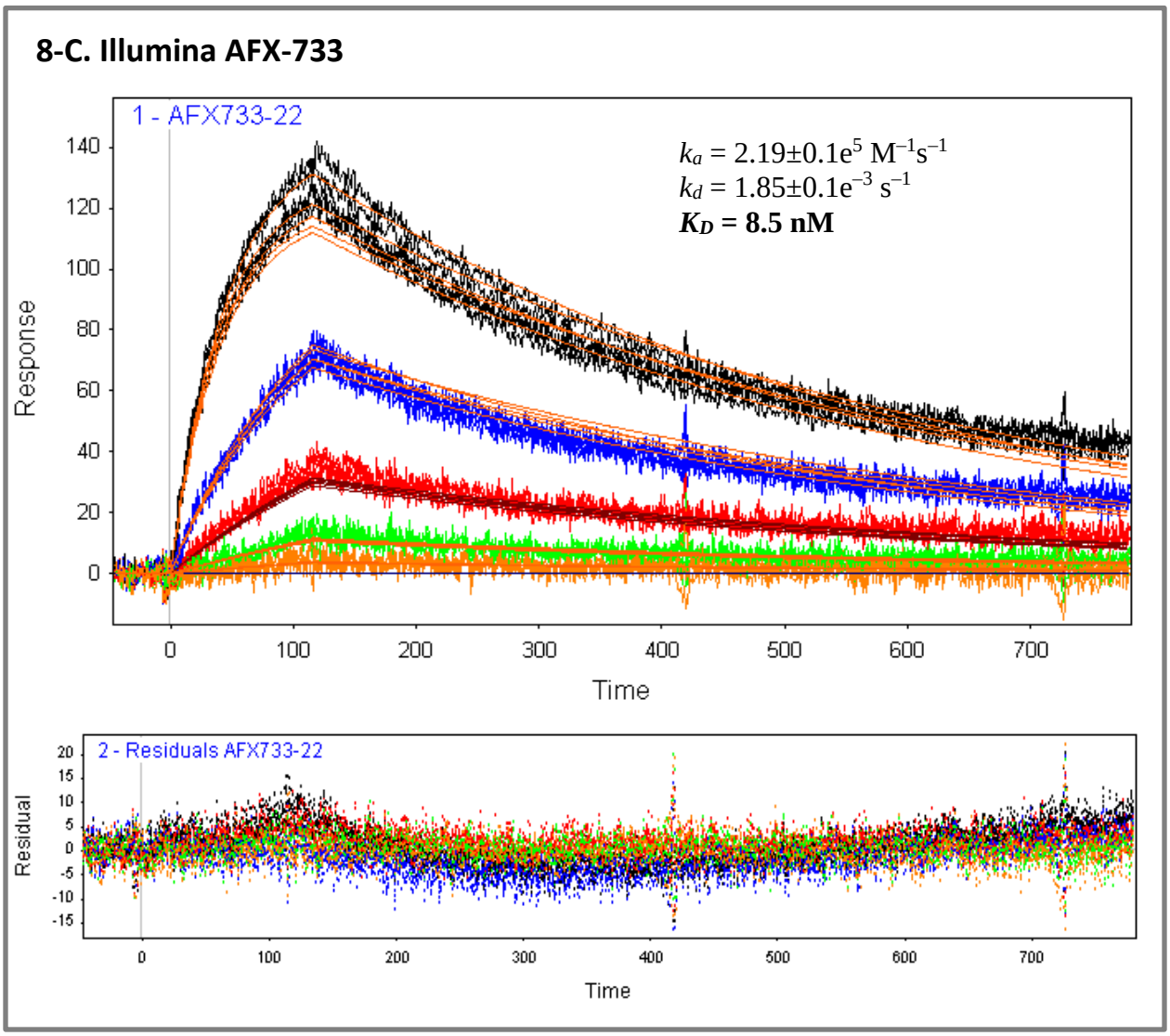


Figure 8. Comparison of the kinetic fits with residuals of the MS2 antibodies, determined using a Proteon XPR36 system. **A:** Kinetics of Illumina affinity-enhanced MS2 scFv (AFX-719; 61.2 pM). **B:** Kinetics of original MS2 scFv (15.5 nM). **C:** Kinetics of Illumina thermostable-enhanced MS2 scFv (AFX-733; 8.66 nM).

4. DISCUSSION

This study established and standardized the parametric tests for performance on the MS2 scFv antibody. This antibody was selected by the DARPA ATP as the initial substrate for performers to use in demonstrating their molecular schemes for improving the thermal stability and affinity of an antibody for its target antigen. The test bed developed was used to define the physical and functional properties of the reference MS2 scFv antibody and establish the baseline for subsequent testing of the engineered antibodies submitted by the ATP performers. A snapshot of the MS2 scFv's physical characteristics was obtained using the NanoDrop, Experion, and DLS measurement platforms. Measurements of the MS2 scFv's

functional characteristics, for assessing the effects of molecular engineering on thermal stability and affinity, were obtained using the DSC, ELISA, and SPR analytic platforms.

An accurate assessment of protein concentration is critically important for all of the test procedures described in this report. We applied the standard technique of spectrophotometry with the NanoDrop ND-1000 system. This instrument was employed to provide the A_{280} value of the sample, which is influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient was used in conjunction with the A_{280} value to determine an accurate concentration.

After concentration was determined via spectrophotometry with the NanoDrop system, molecular weight and purity data were collected with the Experion automated electrophoresis system. This system employs microfluidic technology to automate electrophoresis for protein analysis. The results of Experion analysis of the MS2 scFv protein fell within the acceptable range of purity for use in assay development, and the molecular weight determined by the software (shown in Figure 1) was typical for an scFv.

DLS was used in conjunction with the Experion and NanoDrop systems to illustrate how the protein behaved in solution. DLS data indicate the physical state and potential aggregation of a protein in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. The DLS data established whether the MS2 scFvs provided by Illumina were monomeric and monodisperse. Less than 1% of the sample mass appeared to be aggregating in solution (Figures 2 and 3). To ensure that all testing would be consistent and to mitigate the exacerbating effect of freeze-thawing on future sample aggregation, the Illumina MS2 scFvs were aliquoted into single-use vials and centrifuged before use.

In the next round of testing, the thermostability of the Illumina MS2 scFvs was evaluated using DSC, ELISA and SPR systems. DSC was used to obtain a quantitative T_m for the MS2 scFvs. The T_m should predict the results of ELISA and SPR thermostability testing. A T_m above 70 °C predicts that the percent activity of the MS2 scFvs after thermal stress should remain above 50%. A T_m below 70 °C predicts at least a 50% decrease in MS2 scFv activity after thermal stress. The Illumina MS2 scFv antibody that was optimized for thermostability (AFX-733) described herein exhibited a T_m of 73.6 °C (Figure 4), compared with a T_m from the original MS2 scFv of 67.5 °C (*4*). Therefore, it was expected that heating this sample above 70 °C would not cause the sample to unfold and lose at least 50% of its activity when evaluated by ELISA and SPR systems.

The results of the thermal stress test demonstrated that the Illumina MS2 scFv remained active for over 60 min of heating at 70 °C. The ELISA and SPR data confirmed that the Illumina scFv (AFX-733) was able to bind the MS2CP, even after 60 min of heating, unlike the original MS2 scFv reference antibody, which was unable to bind the MS2CP after only 15 min of heating at 70 °C (Figures 6 and 7).

SPR was also used to obtain a kinetic analysis of the affinity-enhanced Illumina MS2 scFv (AFX-719) binding to its target antigen MS2CP to compare binding parameters with the original antibody. Kinetic data for AFX-719 MS2 scFv binding to the MS2CP was obtained

using the Proteon XPR36 SPR platform, which yielded a K_D of 61.2 pM, whereas the original MS2 scFv yielded a K_D of 8.66 nM (Figure 8). The affinity K_Ds clearly show that Illumina well exceeded the 100-fold improvement requested by DARPA.

5. CONCLUSION

The DARPA ATP seeks to establish methods for rapidly engineering a given antibody reagent to exhibit physical and functional properties that far exceed those of its native state, and thereby expand user confidence in fielding antibody-based detection and diagnostic platforms in environments or operational scenarios in which currently available reagents exhibit degradation or interference. By optimizing the thermal stability and binding affinity of an antibody for its biological target, the DARPA ATP will develop antibody reagents that can reliably function in harsh environmental conditions and increase the sensitivity of sensor platforms to detect lower levels of threat agents.

This report documents the testing of an improved thermostable antibody (AFX-733) and an affinity-improved antibody (AFX-719), which were both produced by Illumina. This study evaluated the physical and functional characteristics of both scFvs in the ECBC testing pipeline. The results were compared to the baseline characteristics of the original antibody's physical properties, to include concentration, molecular weight, purity, and state of aggregation in solution, and functional measures such as binding affinity and thermal stability. Both antibodies supplied by Illumina exhibited enhanced thermal stability and/or affinity for binding to the MS2CP antigen.

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ACRONYMS AND ABBREVIATIONS

A_{280}	absorbance of light at 280 nm
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ATP	Antibody Technology Program
BGG	bovine γ-globulin
CRP	Critical Reagents Program
DARPA	Defense Advanced Research Projects Agency
DLS	dynamic light scattering
DSC	differential scanning calorimetry
ECBC	U.S. Army Edgewood Chemical Biological Center
ELISA	enzyme-linked immunosorbent assay
HRP	horseradish peroxidase
JPMO BSV	Joint Product Management Office for Biosurveillance
k_a	association rate constant
k_d	dissociation rate constant
K_D	affinity constant
MDB	milk diluent block
MS2CP	MS2 coat protein
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with 0.05% Tween 20
R_{Max}	maximum analyte-binding capacity of the surface, in response units
RU	response unit
scFv	single-chain fragment variable
SPR	surface plasmon resonance
T_m	melting temperature

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